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# The epidermal differentiation-associated Grainyhead gene Get1/Grh13 also regulates urothelial differentiation

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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

09 February 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see all three referees consider the study as being an interesting one in principle. Still they all raise a number of major concerns that need to be addressed by further experimentation. We will thus be able to consider a revised manuscript if you can address the referees' criticisms in an adequate manner. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

## REFeree REPORTS

Referee #1 (Remarks to the Author):

Yu and colleagues have used array/bioinformatics approaches to compare transcriptional regulator induction during barrier development in two diverse epithelia - epidermis and bladder epithelia - producing extremely useful and fascinating data. Their focus on Get1 reflects the presence of the knockout mice in their lab (several previous publications) and is rationalised as a transcription factor regulating two very different types of barrier formation - ie the transcription factor must have different targets in each type of epithelia. They provide epigenetic evidence to build an explanation

for how one transcription factor can regulate two different types of epithelial terminal differentiation.

1. the immunohistochemistry of Fig. 3 a, b is not very convincing and could be improved - also why is in situ hybridisation used at E15.5 and immunohistochemistry used at E18.5 in the same figure (Supplementary Fig)?

2) When searching for candidate sites within Uroplakin promoters I disagree with the view that they have to be in "evolutionarily conserved" locations -

eg Odom DT, Dowell RD, Jacobsen ES, Gordon W, Danford TW, Macisaac KD, Rolfe PA, Conboy CM, Gifford DK, Fraenkel E (2007) Tissue-specific transcriptional regulation has diverged significantly between human and mouse. *Nat Genet* 39: 730-732

Wilson MD, Barbosa-Morais NL, Schmidt D, Conboy CM, Vanes L, Tybulewicz VL, Fisher EM, Tavarè S, Odom DT. Species-specific transcription in mice carrying human chromosome 21 *Science* 2008, 322:434-8.

and other publications stressing rapid change/lack of conservation in cis-regulatory regions.

If Get1 is directly regulating uroplakins it must have a binding site in the other uroplakin promoters. If it does not, then it is likely Get1 is affecting some other transcription factor and indirectly regulating the uroplakins.

Are there potential binding sites in the other promoters? Could a quick gel-shift or ChIP be used to demonstrate that Get1 widely regulates the Uroplakins?

3) if the authors are correct that Get1 regulates terminal differentiation in two diverse epithelia then is it regulating terminal differentiation in all barrier-forming epithelia? - could the authors look at gut or another differentiating epithelia in their knockout and by immunohistochemistry? - so they can strengthen and broaden their argument?

Minor

Fig. 1 - Sprr13 is now Lce1A1 - Brown et al, *JID*, 2007

I think the nomenclature should be corrected given the recent publicity (*Nature Genetics* January 2009, two publications) showing Lce genes are psoriasis susceptibility loci in human. The authors in a previous publication showed major and specific change to epidermal Sprr1 genes in response to Get1 knockout but unfortunately few readers will make the connection to Lce genes because the old nomenclature is used.

Referee #2 (Remarks to the Author):

Yu et al identified a role of Get1 in barrier function in the urothelium, which is consistent with its known role in epidermal barrier function. Interestingly, however, the effect of Get1 on barrier function in both tissues is mediated through different target genes, which are expressed in a tissue-specific manner. The authors further show that uroplakin II is a direct target of Get1. These results are interesting for the field. Specific criticisms/questions are listed below:

Specific points:

1.) The data presented in Fig.1 and Fig.2 are entirely based on microarray results. At least the expression pattern of one gene in each cluster should be verified by quantitative RT-PCR.

2.) The immunostainings shown in Fig.3A and B are of rather poor quality. One has the impression that there are also stained cells in the mesenchyme (is this real?) and weaker staining is also seen in some basal cells of the E18.5 animals. A negative control (second antibody only or even better first antibody after blocking with the immunization peptide) should be included. Ideally, immunofluorescence should be used - this would allow double staining with antibodies against Get1 and Uroplakin II. This would further strengthen the in vivo relevance of the Get1- Uroplakin II

connection.

3.) The percentage of pH3-positive cells (Fig.5K,L) should be quantified. It seems surprising that the proliferating cells are located in the most upper layer and not in the basal layer - please comment.

4.) Fig.7A and B: These results should be replaced by results from Real-Time RT-PCR. In addition, the down-regulation of claudins should be verified by Real-Time RT-PCR, since this could also contribute to the defect in barrier function.

5.) Fig.7B: How was the transfection efficiency controlled? Normalization?

6.) Fig. 8D,E: It would be more important to show protein data (western blotting using antibodies against Get1 and uroplakin II).

7.) Fig. 8F: The original data should be shown. How often was this experiment performed? Error bars should be added.

Minor issues:

8.) The last part of the Introduction is more or less a repetition of the abstract - this could be significantly shortened. The discussion could also be shortened, since there is a lot of repetition of the results.

9.) Immunostainings and histological pictures: please provide magnification

Referee #3 (Remarks to the Author):

#### Overview

Using genechip array data, the authors examine expression of differentiation-associated transcriptional regulators by skin and bladder during mouse development. One of these transcriptional regulators, Get1, is expressed in the differentiation-associated intermediate and superficial cells of mouse bladder epithelium. Get1 knockout mouse bladder epithelium is shown to develop a poorly differentiated bladder epithelium with compromised barrier function and this was shown to be associated with reduced expression of urothelial differentiation-associated cytokeratins and uroplakins. The authors present evidence that Get1 is involved in transcriptional regulation of UPK2 gene expression using the RT4 human bladder cancer cell line. Although Get1 is expressed in both differentiated skin and bladder epithelium, UPK2 is not induced by Get1 in epidermal keratinocytes and this is indicated to be due to different histone modifications between the two tissues. Thus, the evidence is supportive of a role for Get1 in transitional and stratified squamous epithelial programs, but suggests that further mechanisms are involved in tissue-specific development.

#### Major points:

1. The title to the manuscript is somewhat misleading as the effect of Get1 on epidermal differentiation is not specifically investigated here.
2. The authors perform a genome-wide gene expression study using genechip arrays, but none of the data is confirmed by real time quantitative RTPCR or western blot analysis, leading the authors to over-interpret and to draw unsupported assumptions from the cluster analysis of the genechip data. It is critically important that the authors confirm the transcript and protein expression of (at least) Get1 in bladder and skin development/differentiation, as this gene is central to the paper. The authors do not justify why the genearray data led them to select Get-1 for further study over all the other transcriptional regulators that are common or differentially-expressed during skin and bladder differentiation and this rather makes the genechip data redundant.
3. The selection of data for figures does not always represent the most critical or relevant information. For example, Figure 1B would be more informative if it included expression of skin differentiation markers by bladder (and visa versa). Figures 1E and 1F are not particularly informative. Figure 2 does not seem necessary, as the data is not used to address any further questions in the paper and could be included as supplementary data.

4. Figure 3 is critical to the paper, but is not of sufficient quality. In particular, the Get1 localisation does not look particularly specific to the urothelium. Get1<sup>-/-</sup> bladder tissue labelled with the Get1 antibody should be included as a specificity control in the immunohistochemical studies. IHC for uroplakins would also be informative here. There is a strange mix of WT and KO mice from E16.5 and E18.5 stages represented in Figure 3.

Supplementary figure S2 serves only to add to the confusion about where the Get1 transcript and protein is expressed in the bladder and associated urinary tract.

5. The introduction does not represent the literature sufficiently. It is implied that the transcriptional mechanisms of urothelial differentiation remain to be discovered. The authors have not included work by Oottamasathien et al. (Dev Biol 2007, 304, 556-566) which investigates differentiation of ES cells into bladder tissue and the TFs involved, also Varley et al. (Cell Death Diff 2009, 16, 103-114) which demonstrates that PPAR  $\alpha$ -induced expression of IRF-1 and FOXA1 TFs directly regulate the urothelial differentiation programme (including uroplakin expression).

Results and discussion of the paper should not be included in the Introduction.

6. The authors have not considered that their tissue preparations (eg for gene arrays) will have included stromal tissues, rather than representing epithelial-only preparations.

7. In the results, it is stated that the genechip arrays demonstrated all the members of the uroplakin family are significantly downregulated in Get1<sup>-/-</sup> bladders, but the full data needs to be shown. There seems to have been very little attempt to develop any continuity in the findings from Figure 1 and 6. The authors suggest that Get1 has a direct transcriptional regulatory effect on UPK2 gene, but in UPK2 knockout mice, an up-regulation of the other UPK genes was seen (Kong XT et al., Roles of uroplakins in plaque formation, umbrella cell enlargement, and urinary tract diseases JCB, 2004, 1195-1204). This is not discussed, but might suggest that the primary effect of Get1 on epithelial differentiation is further upstream.

8. As Get1 has been implicated in cell:cell adhesion in the epidermis and similar observations were also noted in the bladder, it would seem that this could be significant in the inhibition of terminal epithelial differentiation in the Get1 knockout mouse. It is therefore unclear why this has been demoted to supplementary data, although real-time RT-qPCR would be preferred.

#### Minor points:

1. The number of replicates is not indicated in the figure legends.
2. In Figure 1B the x-axis for the expression of genes in dorsal skin is incorrectly labelled.
3. In Figure 4G there is no indication of how the area/what size was selected for the counting of the vesicles. Figure 4H is superfluous.
4. In the text Figure 4 should be mentioned in order of A, B, C etc.
5. Figure 5 - methylene blue penetration data is not convincing - need to illustrate this histologically by post-sectioning.
6. Figure 6A is not necessary as it is repeated in the text of the results.
7. Get1<sup>+/-</sup> mice (Results) is presumably a typo.
8. There should be concluding remarks made at the end of the discussion emphasising the importance of the work and putting the findings into context.

1st Revision - authors' response

28 March 2009

We thank you for inviting us to resubmit a revised manuscript, and greatly appreciate the reviewers' enthusiasm for our study. We thank them for their thoughtful reviews and helpful suggestions. We have implemented all suggested changes in our improved manuscript, which we are very pleased to submit. Enclosed are our responses to the reviewers, and explanation of the changes made to the manuscript and figures.

#### Referee #1

1) the immunohistochemistry in Fig. 3 a, b is not very convincing and could be improved - also why is in situ hybridisation used at E15.5 and immunohistochemistry used at E18.5 in the same figure (Supplementary Fig)?

*We have improved the quality of the Get1 staining by lowering secondary antibody concentration. New panels have been placed in the figure (currently Fig 1A&B). Multiple experiments have shown that Get1 is expressed in umbrella cells. The in situ hybridization experiments for Get1 in mouse bladder, and the immunolocalization for Get1 protein in ureter epithelium, are not critical for our paper, and these supplementary data have been removed from the revised manuscript.*

2) When searching for candidate sites within Uroplakin promoters I disagree with the view that they have to be in "evolutionarily conserved" locations. If Get1 is directly regulating uroplakins it must have a binding site in the other uroplakin promoters. If it does not, then it is likely Get1 is affecting some other transcription factor and indirectly regulating the uroplakins. Are there potential binding sites in the other promoters? Could a quick gel-shift or ChIP be used to demonstrate that Get1 widely regulates the Uroplakins?

*The reviewer makes an excellent point. To address this concern, we analyzed potential Get1 DNA binding sites in the uroplakin genes (Upk1a, Upk1b, Upk2, Upk3a, Upk3b) using BEARR (Batch Extraction and Analysis of cis-Regulatory Regions) and the VISTA Genome Browser. We identified potential Get1 binding sites with PWM scores > 5.7 that are located in a genomic region from 50kb upstream and 25kb downstream of the start sites. Multiple high affinity, but non-conserved, Get1 DNA binding sites were found in all uroplakin genes. The ability of several of these sites to bind Get1 was tested in EMSA assays, and we found that many are indeed capable of binding Get1. In addition to the Upk2 site, which is the focus of our study, we found two conserved potential Get1 binding sites in the far upstream regions of Upk1b (approx -14kb) and Upk3a (approx -7kb), allowing us to test in vivo Get1 binding in human bladder epithelial cells. We used ChIP assays to demonstrate that these sites are occupied by Get1 in human urothelial cells. The EMSA and ChIP data are shown in Supplementary Figure S6 and referred to in the manuscript as suggesting that Get1 may directly regulate other uroplakin genes in addition to Upk2.*

3) if the authors are correct that Get1 regulates terminal differentiation in two diverse epithelia then is it regulating terminal differentiation in all barrier-forming epithelia? - could the authors look at gut or another differentiating epithelia in their knockout and by immunohistochemistry? - so they can strengthen and broaden their argument?

*Our previous studies showed that Get1 is highly expressed in other developing internal epithelia, including stomach and small intestine (Kudryavtseva et al, Dev Dyn 2003). We showed impaired differentiation of the forestomach in a previous paper (Yu et al, Dev Biol 2006). We have also noticed marked abnormalities in the small intestine of Get1<sup>-/-</sup> mice, which may be consistent with a barrier abnormality. These observations are now mentioned and we have included a Supplementary Figure (S7) showing histology of stomach and small intestine. While these abnormalities need to be further characterized, and are outside the focus of this manuscript, we mention these findings as a support for the role of Get1 in additional internal epithelia.*

4) Fig. 1 - Sprl3 is now Lce1A1 - Brown et al, JID, 2007 I think the nomenclature should be corrected given the recent publicity (Nature Genetics January 2009, two publications) showing Lce genes are psoriasis susceptibility loci in human. The authors in a previous publication showed major and specific change to epidermal Sprl genes in response to Get1 knockout but unfortunately few readers will make the connection to Lce genes because the old nomenclature is used.

*We thank the referee for this suggestion. We corrected Sprl3 to Lce1A1 in Fig. 1. As Referee #3 suggested, we have put the original Figure 1E and F into a Supplementary Figure (S2).*

Referee #2

1.) The data presented in Fig.1 and Fig.2 are entirely based on microarray results. At least the expression pattern of one gene in each cluster should be verified by quantitative RT-PCR.

*We agree with the referee and have verified the expression patterns of multiple genes in Figure 1 and 2 by qRT-PCR. These data are shown in Supplementary Figures S1 and S3. Note that as Referee #3 suggested, we have put the original Figure 2 into supplementary data (currently Supplementary Figure S3).*

2.) The immunostainings shown in Fig.3A and B are of rather poor quality. One has the impression that there are also stained cells in the mesenchyme (is this real?) and weaker staining is also seen in some basal cells of the E18.5 animals. A negative control (second antibody only or even better first antibody after blocking with the immunization peptide) should be included. Ideally, immunofluorescence should be used - this would allow double staining with antibodies against Get1 and Uroplakin II. This would further strengthen the in vivo relevance of the Get1- Uroplakin II connection.

*See response to comment 1 by Referee #1. There is minimal background staining in the mesenchyme of E16.5 bladders. We are confident that this represents background staining and previous in situ hybridization studies indicate that the Get1 expression is limited to the epithelial cells of the bladder. Furthermore, similar background staining was observed in Get1 knockout bladders.*

3.) The percentage of pH3-positive cells (Fig.5K, L) should be quantified. It seems surprising that the proliferating cells are located in the most upper layer and not in the basal layer - please comment.

*The quantification of pH3 positive cells, which is based on 4 WT and 4 Get1 knockout bladders at E16.5, has been added as panel O in Figure 4. At birth, proliferating cells are normally located in superficial cell in mouse bladder epithelium (Erman A, et al. Histochem Cell Biol 121, 63-71, 2004).*

4.) Fig.7A and B: These results should be replaced by results from Real-Time RT-PCR. In addition, the down-regulation of claudins should be verified by Real-Time RT-PCR, since this could also contribute to the defect in barrier function.

*We have validated the uroplakin downregulation in Get1 knockout bladders with Q-PCR. This data, along with several other genes, is shown in Figure 5C. Q-PCR validation of claudin mRNAs is shown in Supplementary Figure S5.*

5.) Fig.7B: How was the transfection efficiency controlled? Normalization?

*We assume that the referee is referring to previous Fig. 8B. To control for transfection efficiency, transfections were normalized to Renilla luciferase vector as previously described (Lu et al, Oncogene 2006). This has been added to the Materials and Methods.*

6.) Fig. 8D, E: It would be more important to show protein data (western blotting using antibodies against Get1 and uroplakin II).

*We used immunostaining with Get1 and Upk2 antibodies to show that while Get1 protein is expressed in both RT4 cells and differentiated human keratinocytes, Upk2 protein is selectively expressed in RT4 cells. This data is in Figure 8F.*

7.) Fig. 8F: The original data should be shown. How often was this experiment performed? Error bars should be added.

*This data is now in Fig. 8G. In addition to quantification by Q-PCR (error bars included), we now show the PCR products from IPs on an agarose gel. This experiment has been repeated four times, and two distinct experiments are shown in the figure (Q-PCR based and direct gel).*

8.) The last part of the Introduction is more or less a repetition of the abstract - this could be significantly shortened. The discussion could also be shortened, since there is a lot of repetition of the results.

*We agree with the referee and have removed repetitive text and shortened the Introduction.*

9.) Immunostainings and histological pictures: please provide magnification

*We have included magnification to the immunostaining and histological pictures.*

## Referee #3

1. The title to the manuscript is somewhat misleading as the effect of Get1 on epidermal differentiation is not specifically investigated here.

*The reviewer is correct that epidermal differentiation is not specifically investigated in this paper. However, the role of Get1 in epidermal differentiation is extensively studied, and we make this reference in the title because we feel that the fact that Get1 plays analogous roles in both epithelia is of great interest. Therefore, we would suggest keeping the title as is. However, we would be happy with alternative and more restricted titles such as: "The Grainyhead-like factor Get1/Grhl3 participates in regulation of urothelial differentiation".*

2. The authors perform a genome-wide gene expression study using genechip arrays, but none of the data is confirmed by real time quantitative RTPCR or western blot analysis, leading the authors to over-interpret and to draw unsupported assumptions from the cluster analysis of the genechip data. It is critically important that the authors confirm the transcript and protein expression of (at least) Get1 in bladder and skin development/differentiation, as this gene is central to the paper. The authors do not justify why the genearray data led them to select Get-1 for further study over all the other transcriptional regulators that are common or differentially-expressed during skin and bladder differentiation and this rather makes the genechip data redundant.

*The referee makes a good point about the need for validation of microarray data, and while it is impossible to validate all the microarray data, the revised manuscript contains multiple Q-PCR experiments to validate key findings. These data are in Figure 5 and Supplementary Figures S1, S3, and S5. Also, we show protein expression of Get1 in mouse bladder epithelium and in cell lines. The microarray datasets will be publicly available, and likely to be used by investigators to make significant contributions to our understanding of genes important in bladder and epidermis differentiation. Since Get1 was one of the transcription factors expressed in both epithelial tissues, we took advantage of Get1 knockout mice made earlier in our laboratory to investigate does it has similar effect in bladder.*

3. The selection of data for figures does not always represent the most critical or relevant information. For example, Figure 1B would be more informative if it included expression of skin differentiation markers by bladder (and visa versa). Figures 1E and 1F are not particularly informative. Figure 2 does not seem necessary, as the data is not used to address any further questions in the paper and could be included as supplementary data.

*We have added the microarray data for expression of skin differentiation markers in bladder, and bladder differentiation marker in skin in Supplementary Figure S1A. We have placed Figure 1E and F (currently Supplementary Figure S2B and C), as well as Figure 2 (currently Supplementary Figure S3A) into supplementary results.*

4. Figure 3 is critical to the paper, but is not of sufficient quality. In particular, the Get1 localisation does not look particularly specific to the urothelium. Get1<sup>-/-</sup> bladder tissue labelled with the Get1 antibody should be included as a specificity control in the immunohistochemical studies. IHC for uroplakins would also be informative here. There is a strange mix of WT and KO mice from E16.5 and E18.5 stages represented in Figure 3. Supplementary figure S2 serves only to add to the confusion about where the Get1 transcript and protein is expressed in the bladder and associated urinary tract.

*See response to comment 1 by referee #1. There is some background staining in the E16.5 bladder but it is the same in the knockout mice, and in situ hybridization studies indicate that Get1 is selectively expressed in the epithelium. We have removed previous Supplementary Figure S2.*

5. The introduction does not represent the literature sufficiently. It is implied that the transcriptional mechanisms of urothelial differentiation remain to be discovered. The authors have not include work by Ootamasathien et al. (Dev Biol 2007, 304, 556-566) which investigates differentiation of ES cells into bladder tissue and the TFs involved, also Varley et al. (Cell Death Diff 2009, 16, 103-114) which demonstrates that PPAR-gamma;-induced expression of IRF-1 and FOXA1 TFs directly regulate the urothelial differentiation programme (including uroplakin expression). Results and discussion of the paper should not be included in the Introduction.

*We thank the reviewer for pointing this omission out. We now present the work of Oottamasathien et al. (2007) and Varley et al. (2009) in the Introduction and Discussion.*

6. The authors have not considered that their tissue preparations (eg for gene arrays) will have included stromal tissues, rather than representing epithelial-only preparations.

*We make this clear in the Materials and Methods, as well as in the Results. This feature of the experiments has both advantages and disadvantages, and we have been careful to consider this feature in our interpretations. It should be noted that the back skin also contains non-epithelial component (dermis) in addition to epidermis.*

7. In the results, it is stated that the genechip arrays demonstrated all the members of the uroplakin family are significantly downregulated in Get1<sup>-/-</sup> bladders, but the full data needs to be shown. There seems to have been very little attempt to develop any continuity in the findings from Figure 1 and 6. The authors suggest that Get1 has a direct transcriptional regulatory effect on UPK2 gene, but in UPK2 knockout mice, an up-regulation of the other UPK genes was seen (Kong\_XT et al., Roles of uroplakins in plaque formation, umbrella cell enlargement, and urinary tract diseases JCB, 2004, 1195-1204). This is not discussed, but might suggest that the primary effect of Get1 on epithelial differentiation is further upstream

*We have validated the uroplakin microarray data with Q-PCR (Fig. 5C). In addition, we show the microarray results for all uroplakins in Supplementary Table S2). There is good correlation between the two different experiments, further supported by the immunostaining in Figure 7. The referee makes a good point about the difference in uroplakin gene expression in Get1 and Upk2 knockout mice. We agree with the reviewer that this is consistent with a more upstream and primary role for Get1 in uroplakin gene expression. This is further supported by the identification of Get1 binding sites in the other uroplakin genes (Supplementary Figure S6). We added the referee's point to the Discussion.*

8. As Get1 has been implicated in cell:cell adhesion in the epidermis and similar observations were also noted in the bladder, it would seem that this could be significant in the inhibition of terminal epithelial differentiation in the Get1 knockout mouse. It is therefore unclear why this has been demoted to supplementary data, although real-time RT-qPCR would be preferred.

*In the revised version, we have combined previous supplementary Figures S3 and Supplementary Table S2 to create Figure 6. Some of the claudins were verified by real time RT-qPCR, shown in Supplementary Figure S5.*

Minor points:

1. The number of replicates is not indicated in the figure legends.

*This information is included either in the Materials and Methods or the Figure legends.*

2. In Figure 1B the x-axis for the expression of genes in dorsal skin is incorrectly labelled.

*We have corrected the mislabeling.*

3. In Figure 4G there is no indication of how the area/what size was selected for the counting of the vesicles. Figure 4H is superfluous.

*Same size area of superficial cells was counted in TEM images from WT and knockout bladders. There is a striking and obvious difference that can also be seen in the images in the new Figure 6. We agree that panel H makes a simple point but it does not take up space and facilitates quick understanding of the data.*

4. In the text Figure 4 should be mentioned in order of A, B, C etc.

*The order has been fixed (currently Figure 3).*

5. Figure 5 - methylene blue penetration data is not convincing - need to illustrate this histologically by post-sectioning.

*We have added the histological methylene blue penetration data into the Figure 5 C and D. The results are based on 4 WT and 4 KO bladders.*

6. Figure 6A is not necessary as it is repeated in the text of the results.

*We agree with the referee, and put this panel into Supplementary Figure S4.*

7. Get1+/- mice (Results) is presumably a typo.

*In some experiments we have used heterozygous mice as controls; they are no different from the WT mice.*

8. There should be concluding remarks made at the end of the discussion emphasising the importance of the work and putting the findings into context.

*Thank you for this suggestion. We have added a concluding statement to the revised version of the paper.*

Again, we greatly appreciate the reviewer's expertise and outstanding suggestions, and hope that the paper can now be accepted for publication in EMBO J.

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2nd Editorial Decision

16 April 2009

Thank you for sending us your revised manuscript. Our original referees have now seen it again. In general, the referees are now positive about publication of your paper. Still, referee 3 feels that there are a few minor issues that need to be addressed (see below) before we can ultimately accept your manuscript. I would therefore like to suggest dealing with the issues raised in an amended manuscript.

Furthermore, there is one editorial issue that needs attention:

Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) to ask for the original scans (for our records).

In the case of the present submission there is one panel that does not fully meet these requirements: Figure 8G.

I therefore like to kindly ask you to send us a new version of the manuscript that contains a suitably amended version of this figure. I feel that it would also be important to explain the assembly procedure for this figure in the figure legend (i.e. that all lanes come from the same gel). Please be reminded that according to our editorial policies we also need to see the original scan for the figure in question.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final print version of the paper.

Thank you very much for your cooperation.

We thank you very much for the positive consideration; the reviewers' expertise and suggestions are highly appreciated. We have replaced the assembled Fig 8G with a new panel where the samples were loaded such that no rearrangement of lanes was necessary (see explanation below). In addition, we have addressed all of Reviewer #3's remaining concerns, including the inclusion of a knockout control in Fig. 2 (panels A-C). We hope that the manuscript can now be accepted for publication in EMBO Journal.

#### The assembled Figure 8G

The previous Figure 8G was assembled from 2 original gels, which contained other lanes and controls that were not included in the final figure. In addition, the order of samples was not optimal for presentation. To adjust the order of lanes, and to remove non-relevant lanes, we had to crop and re-assemble the panels. To avoid any questions, we have repeated the experiment and run the gel in the same order we selected for presentation. The results are the same as before, and the new panel (Fig. 8G) is included in the revised manuscript.

#### Referee #3

1. The referee suggests this title: The epidermal differentiation-associated Grainyhead-like factor Get1/Grh13 is also involved in urothelial differentiation.

*We are happy to go along with the reviewer's suggestion and have replaced the old title in the revised manuscript.*

2. Figure 2 (was Figure 3). The authors have still not included sufficient controls for the Get1 immunohistochemistry as outlined by referee 2 and 3. The most suitable specificity control is bladder tissue from the Get1<sup>-/-</sup> mouse at the equivalent stages.

*We have included the Get1 immunohistochemistry in E18.5 Get1<sup>-/-</sup> bladder as a control in the revised Figure 2 (this now becomes panel 2C). No Get1 immunostaining is observed in the Get1<sup>-/-</sup> bladder.*

3. The authors have cited Oottamasathien et al. (2007) as suggested by the reviewer 3, but clearly have not read the paper. This has led to inappropriate citing of the reference in the Introduction and an inaccurate claim on page 3 of the Discussion.

*We have appropriately cited Oottamasathien et al's work in the introduction (by lumping the citation together with another citation, we may have inadvertently given too much credit to this paper, which simply showed expression of two transcription factors in differentiated bladder).*

*As to the discussion, we assume that the reviewer is referring to our claim that "Get1 is the first transcription factor in vivo demonstrated to promote to promote terminal differentiation of bladder epithelium". While we believe this is true, we have deleted a reference to "the first".*

4. Results section "Epigenetic mechanisms for the cell-specific ...." The claim that RT4 cells express Get1 and UpkII similar to normal bladder cells is unsubstantiated.

*We agree with the reviewer, and have removed "similar to normal bladder cells" in the revised manuscript.*

5. Supplementary Figure 1A. The graph is labelled "normalized mRNA level", but this data is from the Affymetrix chips, so therefore is not strictly mRNA levels. Sequences or assays have not been included for the Nfix and Smarcd3.

*We have fixed the mislabeling of supplementary Figure 1A, and have included the assay IDs for Nfix, Smarcd3 and all other Taqman assays used in the revised manuscript.*

6. Discussion. The authors state that activation of PPAR $\gamma$  induces expression of UPK2 and UPK1b, when in fact this should be UPK2 and UPK1a.

*The typo has been fixed in the revised manuscript.*